(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 310 949 A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 88116110.3

(22) Date of filing: 29.09.88

(51) Int. Cl.⁴: C12P 13/06 , C12P 41/00 , //(C12P13/06,C12R1:13)

(30) Priority: 07.10.87 JP 253127/87

(43) Date of publication of application: 12.04.89 Bulletin 89/15

(84) Designated Contracting States: CH DE FR GB IT LI SE

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(54) Process for producing D-alanine.

(57) It is possible to produce and accumulate D-alanine selectively and to improve the amount of production and accumulation of D-alanine by cultivating a microorganism having both an ability to produce D-alanine and a resistance to D-cycloserine and belonging to the genus Brevibacterium.

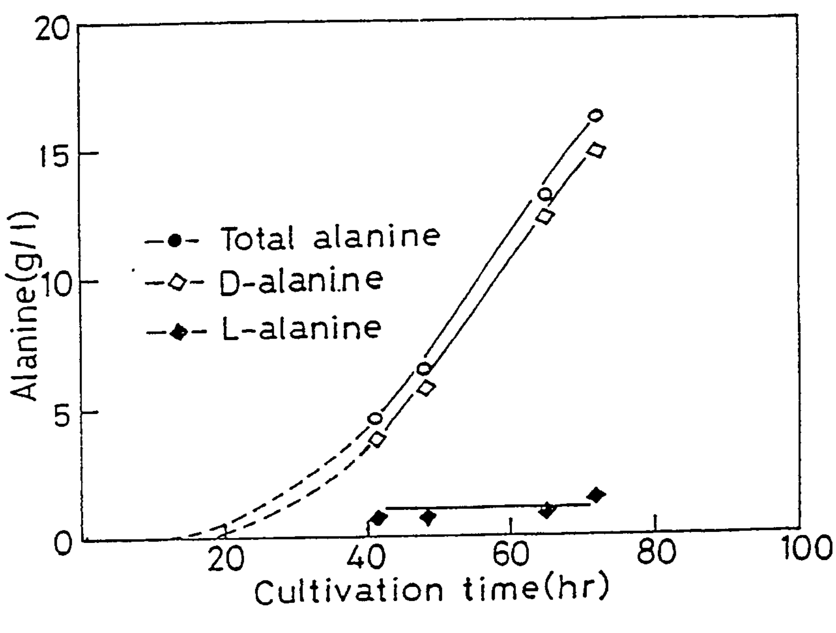

Figure 1

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PROCESS FOR PRODUCING D-ALANINE

Background of the Invention

(1) Field of the Invention

The present invention relates to an industrially favorable process for producing D-alanine.

D-alanine is an unnatural amino acid and it is a useful compound for a reagent as it is or for a raw material for synthesizing peptides and so on. Accordingly, the demand has been recently increasing.

(2) Description of the Prior Art

A number of processes for producing DL-alanine by means of a fermentation process using a microoganism belonging to the genus Brevibacterium have been hitherto known. An improved process for producing DL-alanine by using a microorganism belonging to the genus Brevibacterium and having sensitivity against threonine and methionne, also has been known (J.Gen. Appl. Microbiol. 1971, 17(2), 169-72).

However, it is inevitable to use a certain optical resolution operation for obtaining only D-alanine, because L-alanine is also produced by these process.

Namely, it has never been achieved yet to produce D-alanine by fermentation with low cost, simply and industrially favorably.

Summary of the Invention

One of the purposes of the present invention is to offer a process for producing D-alanine by means of an optically selective fermentation method.

The second purpose of the present invention is to offer a process for increasing the accumulative concentratien of D-alanine by fermentation using one microorganism.

The third purpose of the present invention is to offer a process for producing D-alanine without using an optical resolution operation.

The fourth purpose of the present invention is to offer a process for producing D-alanine with low cost and simply.

The final purpose of the present invention is to offer an industrially favorable process for producing D-alanine.

Other and further objects, features and abvantages of the present invention will appear more fully in the following description.

These purposes can be achieved by a process for producing D-alanine characterized by cultivating a microorganism, which belongs to the genus Brevibacterium and has an ability to produce D-alanine and a resistance to D-cycloserine, producing and accumulating thereby D-alanine in a culture medium, and obtaining D-alanine from the culture broth.

Brief Description of the Drawings

Figure 1 shows the quantities of D- and L- alanine, respectively, accumulated in the culture medium with the passage of cultivation time.

Figure 2 shows the quantities of D- and L- alanine, respectively, accumulated in the culture medium with the elapse of cultivation time.

Description of the Preferred Embodiments

The microorganisms used in the present invention are those microorganisms that have an ability to produce D-alanine and a resistance to D-cycloserine and belong to the genus Brevibacterium. Whenever any microorganism belonging to the genus Brevibacterium has these characteristics, it can be included in the range of the present invention even if it has other auxotrophy and drug resistance.

Microorganisms which belong to the genus Brevibacterium and have resistance to D-cycloserine can produce and accumulate D-alanine selectively with high accumulation quantity.

As the representative samples of the microorganisms used in the present invention, for example, Brevibacterium lactofermentum DCSR-26, Brevibacterium lactofermentum DCSR17-2 and Brevebadterium flavum DCSR 2-2 can be cited. Brevibacterium lactofermentum DCSR-26 is derived from Brevibacterium lactofermentum ATCC 13869 (requiring biotin for the growth) and it is a variant having resistance to D-cycloserine. Brevibacterium lactofermentum DCSR17-2 is derived from Brevibacterium lactofermentum DCSR-26 and it is a variant having a further higher resistance to D-cycloserine. Brevibacterium flavum DCSR 2-2 is derived from Brevibacterium flavum ATCC 13826 and it is a variant having a resistance to D-cycloserine.

Brevibacterium lactofermentum DCSR-26 was deposited to Fermentation Research Institute, Agency of Industrial Science and Technology in Japan (abbreviated as FRI thereinafter) on September 24, 1987 and a registration No. FERM BP-2023 was conferod on it. Brevibacterium lactofermentum DCSR 17-2 was deposited to FRI in Japan on March 9, 1988 and a registration No. FERM BP-2024 was confered on it. Brevibacterium flavum DCSR 2-2 was deposited to FRI in Japan on September 12, 1988 and a registration No. FERM BP-2047 was confered on it.

FERM BP number is the registration number of the deposition to FRI based on Budapest Treaty. All restriction upon availability to the public will be irrevocably removed upon granting of the patent and the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, which is longer.

FRI is located in 1-1, Higashi 1-Chome, Tsukuba, Ibaraki ken, Japan.

Derivatian of the variant can be relatively easily carried out by means of an ordinary mutative treatment. Namely, to obtain variants having resistance to D-cycloserine, parent strains are either irradiated by ultraviolet or treated with mutagenic agents (for example, N-methyl-N′-nitro-N nitrosoguanidine, ethyl methanesulfonate and so on) and strains which are capable of growing distinguishably in comparison with the parent strain can be selected from the culture plates contained D-cycloserine in such a concentration that the parent stain can not grow sufficiently.

D-cycloserine resistant strain in the present invention means the strain gaining stronger resistance than that the parent strain has. The relative growth degree of the D-cycloserine resistant strain is preferably 50% or more when the cultivation is carried out in a culture medium containing D-cycloserine with such a concentration that the relative growth degree of the parent strain becomes 40% or less. Here, the relative growth degree is shown by the percentage of the optical density measured at 660nm of the culture broth containing no D-cycloserine.

As the culture medium used in the present invention, various nutrient resources being widely used in cultivation of microorganisms can be used. For example, as the carbon source, sugars such as glucose, molasses, hydrolysate of starch and so on, organic acids such as acetic acid, benzoic acid and so on, alcohols such as ethanol and so on can be used and as the nitrogen source, ammonium sulfate, ammonium nitrate, ammonium chloride, ammonium phosphate, urea, ammonia and so on can be used. Depending upon the kind of inorganic ammonium salts, inorganic salts such as phosphate, calcium carbonate and so on are required to use with them. Moreover, to improve the growth of microorganisms and to accumulate large amount of D-alanine, it is preferable to add, for example, organic nitrogen sources, vitamins, a small amount of metal ions and so on in the above described culture medium. Consequently, the purpose can be sufficiently achieved by adding usually cheap natural products such as soybean hydrolysate and corn steep liquor.

In the present invention, it is preferable to add D,L-alanine containing L-alanine with any ratio in the culture medium. As the additive D,L-alanine containing L-alanine with any ratio, not only a racemic modification of D,L-alanine but also pure L-alanine can be used. The time of the addition is not specifically restricted during the cultivation, but the early or intermediate phase of the cultivation is the most effective. By adding D,L-alanine, the ability of D-alanine accumulation by the microorganism used in the present invention can be improved and L-alanine is converted into D-alanine by the action of the microorganism. As the result, the amount of production and accumulation of D-alanine is surprisingly improved. The amount of

D,L-alanine to be added is not specifically restricted. The larger the amount added, the higher the amount of accumulation of D-alanine. Usually, it is 100g/ℓ or less.

In the present invention, the above described cultivation of the microorganism is preferably carried out in an aerobic condition such as shaking or stirring with aeration. The cultivation temperature is usually in the range of 20-40°C and especially around 30°C is preferable. The pH of the medium is usually at 5-9 and it is preferable to keep it at about neutral for obtaining high yield. Thus, after cultivation for several days, a remarkable amount of D-alanine is produced and accumulated in the culture broth. After the cultivation isfinished, D-alanine produced can be easily obtained by well known isolation and purification procedures such as ion exchange, adsorption and precipitation procedures without using an optical resolution method.

The present invention will be more clearly understood with reference to the following Experiments and Examples.

Experiment 1

A. (Separation of D-cycloserine resistant strains)

Cells of Brevibacterium lactofermentum ATCC 13869 (requiring biotin for the growth) were treated with N-methy-N′-nitro-N-nitrosoguanidine (300 μg/mℓ, at 30°C for 10 minutes) by means of an usual procedure and then spread on the complete synthetic agar plate containing 50 mg/ℓ of D-cycloserine (the complete synthetic agar plate : 2% of gulcose, 2% of agar, 1% of ammonium sulfate, 0.1% of potassium hydrogen-phosphate, 0.04% of 7 hydrate of magnesium sulfate, 0.05% of sodium chloride, 0.25% of urea, 10 mg/ℓ of 7 hydrate of ferrous sulfate, 10 mg/ℓ of 4 hydrate of manganese (II) sulfate and 50 μg/ℓ of biotin). Then, the incubation was carried out at 30°C for 5-7 days and large colonies appeared were separated by means of colony picking to select a D-cycloserine resistant strain (Brevibacterium lactofermentum DCSR-26).

Another D-cycloserine resistant strain (Brevibacterum flavum DCSR 2-2) was obtained in the same way as the above described procedures such as mutation, cultivation and separation of the strain except that Brevibacterium lactofermentum ATCC 13869 was replaced with Brevibacterium flavum ATCC 13826.

Moreover, cells of Brevibacterium lactofermentum DCSR-26 were treated with N-methyl-N′-nitro-N-nitrosoguanidine (300 μg/ml, at 30°C for 10 minutes) by means of an usual procedure and then spread on the complete synthetic agar plate containing 100 mg/ℓ of D-cycloserine (the complete synthetic medium : 2% of glucose, 2% of agar, 1% of ammonium sulfate, 0.1% of potassium hydrogen-phosphate, 0.04% of 7 hydrate of magnesium sulfate, 0.05% of sodium chloride, 0.25% of urea, 10 mg/ℓ of 7 hydrate of ferrous sulfate, 10 mg/ℓ of 4 hydrate of manganese (II) sulfate and 50 μg/ℓ of biotin). Then, the cultivation was carried out at 30°C for 5-7 days and large colonies appeared were separated by means of colony picking to select a D-cycloserine highly resistant strain (Brevibacterium lactofermentum DCSR 17-2).

B. (The degree of resistance of D-cycloserine resistant strains)

Each strain shown in Table 1 was cultivated with shaking by using a bouillon liquid medium at 30°C for 16 hours and the cells grown were harvested and washed well with sterilized physiological saline. This cells suspension was inoculated in 5 ml of minimal media contained D-cycloserine in concentrations of 0, 10 and 50 mg/ℓ, respectively, (the minimal medium containing 2% of glucose, 1% of ammonium sulfate, 0.1% of potassium hydrogen-phosphate, 0.04% of 7 hydrate of magnesium sulfate, 0.05% of sodium chloride, 0.25% of urea, 10 mg/ℓ of 7 hydrate of ferrous sulfate, 10 mg/ℓ of 4 hydrate of manganese (II) sulfate and 50 μg/ℓ of biotin) and cultivations were continued for 64 hours at 30°C to investigate the degree of growth of each strain. The results were shown in Table 1.

It was clear from the data shown in Table 1 that D-cycloserine resistant strains used in the present invention (Brevibacterium lactofermentum DCSR-26, Brevibacterium lactofermentum 17-2 and Brevibacterium flavum DCSR 2-2) grew without obstruction by D-cycloserine and were resistant to D-cycloserine in comparison with the respective parent strains, Brevibacterium lactofermentum ATCC 13869 and Brevibacterium flavum ATCC 13826.

Table 1

Strain	Relative growth degree (%) Concentration of D-cycloserine added (mg/ℓ) 0	10	50
Parent strain Brevibacterium lactofermentum ATCC 13869	100 (60.0)	36.7 (22.0)	0 (0)
Strain of the present invention Brevibacterium lactofermentum DSCR-26	100 (87.0)	78.4 (68.2)	61.8 (53.8)
Strain of the present invention Brevibacterium lactofermentum DCSR 17-2	100 (92.9)	—	109 (101.7)
Parent strain Brevibacterium flavum ATCC 13826	100 (125.2)	87 (109.0)	82 (103.0)
Strain of the present invention Brevibacterium flavum DCSR 2-2	100 (103.1)	103 (106.6)	107 (110.8)

Notice: Optical densities at 660 nm of culture broths were shown in ( ). The relative growth degree was calculated by using the optical density of the culture broth where no D-cycloserine was added as 100%.

Example 1

Each 40 ml of fermentation medium (pH 7.25) containing 10% of glucose, 1.8% ammonium sulfate, 0.05% of di-potassium hydrogenphosphate, 0.025% of 7 hydrate of magnesium sulfate, 0.001% of zinc chloride, 2% of soybean hydrolysate (Ajinomoto, Tokyo, Japan), 3% of calcium carbonate and 30 µg/ℓ of biotin was poured into 1-ℓ Erlenmeyer flask and sterilized in an autoclave at 120°C for 10 minutes to obtain fermentation medium. Brevibacterium lactofermentum DCSR-26 and its parent strain, Brevibacterium lactofermentum ATCC 13869 were inoculated in a bouillon liquid medium respectively and cultivated with shaking ar 30°C for 5 days. 1% of the above described each culture medium was put into the above described fermentation medium respectively. After shaking at 30°C for 5 days, D-alanine was produced in each culture medium as shown in Table 2.

Table 2

	Strain	Amount of D-alanine accumulated	Purity*
Comparative example	Brevibacterium lactofermentum ATCC 13869	9.2 g/ℓ	76%
Example of the present invention	Brevibacterium lactofermentum DCSR-26	13.2 g/ℓ	100%

$$* \text{ Purity} = \frac{\text{D-alanine (g/ℓ)}}{\text{Total alanine (g/ℓ)}} \times 100\%$$

Total amount of alanine in the fermentation medium was analysed by means of an automatic amino acid analyzer. Analysis of D-alanine was carried out both by means of an enzymatic method using D-amino acid oxidase and by means of high performance liquid chromotography (HPLC) using a column for optical resolution (Sumitomo Chemical Industries, Co., Ltd OA-1000).

The Cells were removed by means of centrifugal separation from 1ℓ of the fermentation broth of

Brevibacterium lactofermentum DCSR-26 to obtain supernatant, which was then decolorized with active carbon powder. The decolorized liquid thus obtained was passed through a column where a strong cation exchange resin "DIAION SK-1B" (H^{+} type) was packed to adsorb D-alanine, which was eluted with 2N aqueous ammonia after washing with water. The eluted fractions of D-alanine were concentrated and ethanol was added to the concentrated liquid to obtain precipetated crystals. 9.5g of D-alanine crystals were obtained by recrystalizing with ethanol.
Specific rotation $[\alpha]_D^{25}$ = -14.2° (C=6, 1N-HCl)

Example 2

1ℓ of the fermentation medium described as in Example 1 without calcium carbonate were put into a jarfermenter and sterilized. Brevibacterium lactofermentum DCSR 17-2 was cultivated in the bouillon liquid medium at 30°C for 16 hours. Then, 40 ml of this culture medium was inoculated in the above described 1-ℓ fermentation medium and fermentation was continued aerobically (1v.v.m., impeller speed : 600r.p.m.) for 3 days at 30°C to produce 14.8g/ℓ of D-alanine in the broth. The relations between the cultivation time and the accumulated amount of alanine for 3 days were shown in Figure 1.

D-alanine was separeted and obtained from 1ℓ of the culture broth by the same procedures as described in Example 1 and 11.8g of D-alanine crystals were thereby obtained.
Specific rotation $[\alpha]_D^{25}$ = -14.2° (C=6, 1N-HCl)

Example 3

40 ml of fermentation medium (pH 7.25) containing 10% of glucose, 3% of ammonium sulfate, 0.05% of potassium hydrogen-phosphate, 0.05% of dipotassium hydrogen-phosphate, 0.025% of 7 hydrate of magnesium sulfate, 2% of soybean hydrolysate, 3% of calcium carbonate and 30µg/ℓ of biotin was poured into 1-ℓ Erlenmeyer flask and sterilized in an autoclave at 120°C for 20 minutes to obtain culture medium. Brevibacterium lactofermentum DCSR 17-2 was inoculated in the fermentation medium thus obtained and cultivated at 30°C for 3 days. 20gl/ℓ of racemic D,L-alanine was therein added and the cultivation was furthermore carried out for 64 hours to produce and accumulate 48.8g/ℓ of D-alanine in the culture broth as shown in Figure 2.

The supernatant liquid obtained by removing cells from 200 ml of this culture broth by means of centrifugal separation was decolorized with active carbon powder. The decolorized liquid was passed through a column where a strong cation exchange resin "DIAION SK-1B" (H^{+} type) was packed to adsorb D-alanine, which was eluted with 2N aqueous ammonia after washing with water. The eluted fractions of D-alanine were concentrated and ethanol was added to concentrated liquid to obtain precipitated crystals. 7.2g of D-alanine crystals were obtained by recrystalizing with ethanol.
Optical purity 99.4%
Specific rotation $[\alpha]_D^{25}$ = -14.2° (C=6, 1N-HCl)

Analysis of D-and L-types of alanine was carried out in the same was as described in Exampls 1.

Example 4

Each 40 ml of fermentation medium (pH 6.5) containing 10% of glucose, 2% of ammonium sulfate, 0.05% of potassium hydrogen-phosphate. 60µg/ℓ of biotin, 1% of sodium chloride, 10 mg/ℓ of 7 hydrate of ferrous sulfate, 20 mg/ℓ of 4 hydrate of manganese (II) sulfate, 0.025% of 7 hydrate of magnesium sulfate and 2% of hot water extract of bean cake was poured into 1ℓ Erlenmeyer flask and sterilized in an autoclave at 120°C for 20 minutes to obtain culture medium. Brevibacterium flavum ATCC 13826 and Brevibacterium flavum DCSR 2-2 were respectively inoculated in the fermentation media thus obtained and shaked at 30°C for 3 day. D-alanine was thereby produced in each culture broth as shown in Table 3.

Table 3

	Strain	Amount of D-alanine accumulated	Purity	D/L ratio*
Comparative example	Brevibacterium flavum ATCC 13826	1.7 g/ℓ	85%	6.96
Example of the present invention	Brevibacterium flavum DCSR 2-2	1.9 g/ℓ	95%	13.4

$$^{*}\ \text{D/L ratio} = \frac{\text{Amount of D-alanine}}{\text{Amount of L-alanine}}$$

As the D/L ratio was larger than 10 in the Example of the present invention, it was possible to separate D-alanine with almost no loss by means of recrystallization.

Claims

1. A process for producing D-alanine characterized by cultivating a microorganism which belongs to the genus Brevibaterium and has an ability to produce D-alanine and a resistance to D-cycloserine, producing and accumulating thereby D-alanine in a culture broth and obtaining D-alanine from the culture broth.

2. A process according to Claim 1, D,L-alanine containing L-alanine with any ratio is added in the culture medium.

3. A process according to Claim 1 or 2, the microorganism is a microorganism belonging to the genus Brevibacterium, the species lactofermentum or flavum.

4. A process according to Claim 3, the microorganisms are those derived from the strain ATCC 13869 and belonging to the genus Brevibacterium, the species lactofermentum.

5. A process according to Claim 3, the microorganisms are those derived from the strain ATCC 13826 and belonging to the genus Brevibacterium, the species flavum.

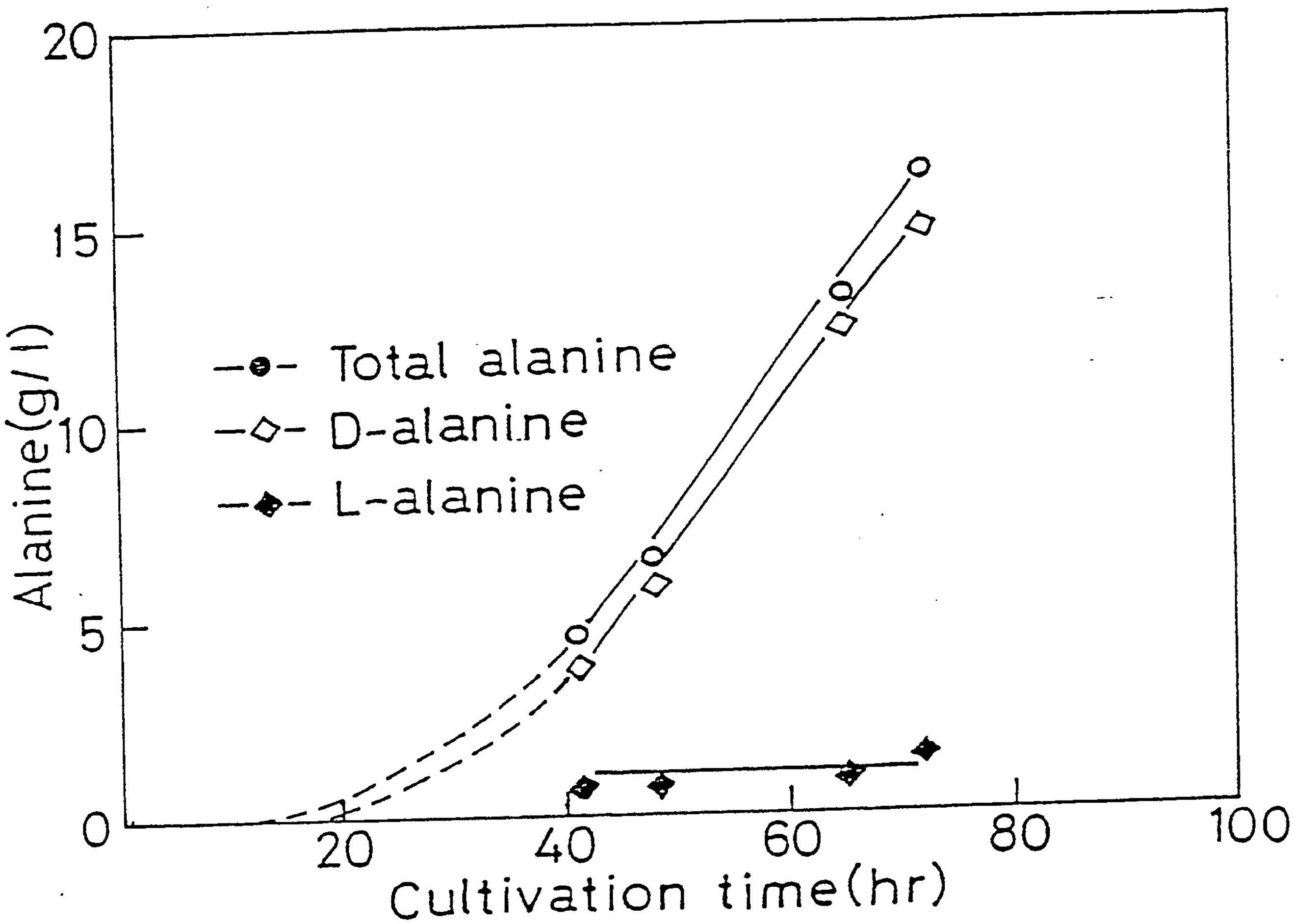
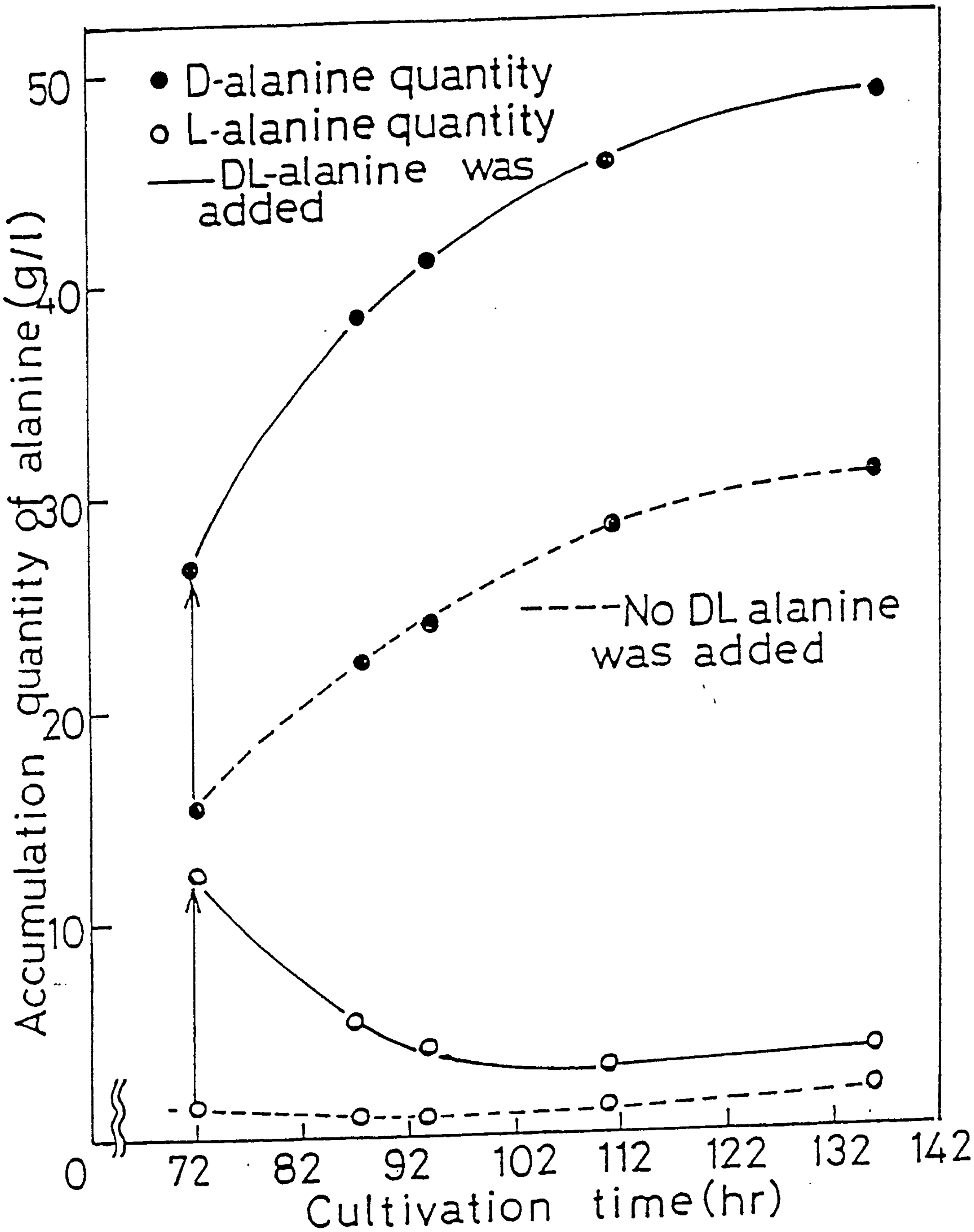

20
15
10
5
0
Alanine(g/l)
Total alanine
D-alanine
L-alanine
20
40
60
80
100
Cultivation time(hr)

Figure 1



• D-alanine quantity
o L-alanine quantity
—— DL-alanine was added
----No DL alanine was added
Accumulation quantity of alanine (g/l)
50
40
30
20
10
0
72
82
92
102
112
122
132
142
Cultivation time(hr)

Figure 2

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(88) Date of deferred publication of the search report: 02.11.89 Bulletin 89/44

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(54) **Process for producing D-alanine.**

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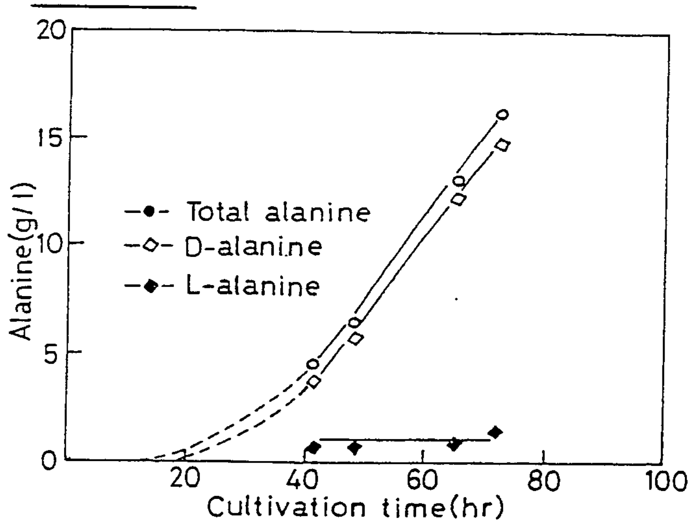

Figure 1

EP 0 310 949 A3

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EUROPEAN SEARCH REPORT

Application Number

EP 88 11 6110

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	CHEMICAL ABSTRACTS, vol. 67, no. 15, 9th October 1967, page 6700, abstract no. 71293s, Columbus, Ohio, US; R.H. REITZ et al.: "The biochemical mechanisms of resistance by streptococci to the antibiotics D-cycloserine and O-carbamyl-D-serine", & BIOCHEMISTRY 6(8), 2561-70(1967) * Abstract * ---	1-5	C 12 P 13/06 // (C 12 P 13/06 C 12 R 1:13)
Y	PATENT ABSTRACTS OF JAPAN, vol. 8, no. 232 (C-248)[1669], 25th October 1984; & JP-A-59 113 893 (NIPPON CARBIDE KOGYO K.K.) 30-06-1984 * Abstract * ---	1-5	
Y	CHEMICAL ABSTRACTS, vol. 87, no. 7, 15th August 1977, page 200, abstract no. 49947z, Columbus, Ohio, US; V.L. CLARK et al.: "D-Cycloserine-induced alterations in the transport of D-alanine and glycine in Bacillus subtilis 168", & ANTIMICROB. AGENTS CHEMOTHER. 1977, 11(5), 877-80 * Abstract * ---	1-5	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
A	US-A-3 871 959 (ICHIRO CHIBATA) ---		C 12 P
A	BIOTECHNOLOGY OF AMINO ACID PRODUCTION, PROGRESS IN INDUSTRIAL MICROBIOLOGY, vol. 24, 1986, pages 3-13, Elsevier, Amsterdam, NL --- -/-		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	04-08-1989	PULAZZINI A.F.R.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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P : intermediate document

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D : document cited in the application
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D,A	J. GEN. APPL. MICROBIOL., vol. 17, no. 2, 1971, pages 169-172; K. SANO et al.: "Microbial production of alanine, L-proline, and L-valine by threonine- or methionine-sensitive mutants of brevibacterium flavum" -----		
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)

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& : member of the same patent family, corresponding document